EFFECT OF RADIOFREQUENCY RADIATION ON mRNA EXPRESSION IN CULTURED RODENT CELLS

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• *Four rodent cell lines were exposed to 2450 MHz microwave radiation at a Specific Absorption Rate (SAR) of 103.5 ± 4.2 W/kg for varying lengths of time at 37°, 40°, 42° and 45° C. mRNA was extracted from microwave-exposed and sham-exposed cells and dot blotted or Northern blotted to nitrocellulose. Radioisotope labelled DNA probes of oncogenes, heat shock protein or long terminal repeat sequences were hybridized to the mRNA, and the resulting autoradiographs analyzed for differences in levels of mRNA expression between exposed and nonexposed samples. With the cell lines and probes used in this study no significant differences in mRNA expression were observed after microwave exposure.*

INTRODUCTION

Radiofrequency radiation (RFR) has been reported to induce adverse effects in biological systems, such as teratogenic and embryo lethal effects in mammals particularly during exposures producing significant hyperthermia[1]. Other studies have implicated microwave exposure with causing changes in chromosome number[2] and structure[3], formation of cataracts in humans[4,5,6], rabbits[7,8,9,10,11] and dogs[12] and promoting malignant tumor formation in rats[13], as well as increasing tumor production and leukemias[14,15].

In addition, microwave exposures have been reported to change the structure of purified double-stranded plasmid DNA, causing it to become nicked and increasing the proportion of relaxed to super coiled molecules[16]. In view of these reports of changes at different levels of cellular function and structure of mammalian systems to microwaves, we asked ourselves if changes at the level of mRNA expression could be detected after microwave exposure of cultured rodent cells. We chose to look at the mRNA expression of certain oncogenes known to show elevated levels during cell replication, at the heat shock proteins, which are known to respond to stresses other than heat[17] and at the long terminal repeat (LTR) region of mouse mammary tumor virus in four rodent cell lines.

MATERIALS AND METHODS

Cell lines. Cell lines used were mouse L5178Y (LY), Chinese hamster ovary (CHO) and two mouse macrophage lines, RAW-264.7 and P388-D1.

In replicate experiments, cells freshly grown to a standard density of 1×10^7/ml were resuspended in 1.0 ml of fresh medium and transferred to a nitrocellulose tube (16 mm × 76 mm) for exposure in the circular wave guide apparatus[18]. CHO, RAW and P388-D1 cells were grown in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum (FCS), while L5178Y cells were grown in Fischer's medium for rat leukemia cells containing 10% heat inactivated FCS.

Exposure conditions. L5178Y, RAW-264.7 and P388-D1 cells were exposed to continuous wave 2450 MHz RFR in the cir-

cular wave guide apparatus of Kiel et al[18]. Using a 1-kW S-band amplifier (Model 10704, MCL La grange, IU) a SAR of 103.5 ± 4.2 W/kg was attained throughout the exposure. Temperature was monitored throughout exposure and in sham-exposed cells by means of Vitek probes in the actual cell suspension. CHO cells were subjected to a SAR half that of the other cell lines, i.e. 51.75 W/kg. All other experimental details were identical to those of the other cell lines. All cells were exposed to microwaves at temperatures of 37°, 40°, 42° and 45°C. At each temperature a series of 6 times of exposure were performed: 0, 3, 5, 10, 15, and 20 min. For each exposure time a sham-exposed control was also performed giving a total of 12 points at each temperature. A room temperature control was substituted at 0 instead of a 0 min reading at the different temperatures in the sham-exposed sets.

After exposure, 4.0 ml of fresh media were added to the cells, and the cells placed in a 37°C CO_2 incubator for 2 hr. After incubation, the cells were pelleted and washed four times in phosphate buffered saline (0.15 M, pH 7.4).

Extraction of RNA. The cell pellet was processed to extract total RNA by the methods of Glison et al[19] and Ullrich et al[20]. If cell pellets were not processed immediately, they were frozen at −70°C for later extraction of RNA. Guanidine thiocyanate solution, 2.5 ml 4M, was added to the cell pellet and rapidly homogenized using a Tekmar Tissumizer. The solution was then transferred to a glass homogenizer and dounced 10-12 times to shear DNA. One gram of solid cesium chloride was dissolved in the guanidine solution before layering the mixture onto an equal volume of 5.7 M cesium chloride solution in a polyallomer tube (13 mm × 51 mm). The gradient was run overnight for 15-16 h in a Beckman SW50.1 rotor using either a Sorvall RC80 or Beckman L8-80 Ultracentrifuge.

After spinning, the pellet was rinsed twice with 70% ethanol, dried by inverting on paper towels, and dissolved in 400 μl of a 20 mM Tris (pH 8.0), 1 mM EDTA solution. Precipitation was effected overnight at −20°C by the addition of 1/10 volume 3M sodium acetate and 2 volumes of ice cold absolute ethanol.

Extraction of mRNA. Total RNA was dissolved in buffer containing 1M lithium chloride and chromatographed using dT cellulose[21]. mRNA was precipitated overnight at −20°C using 1/10 volume 3M sodium acetate and 2 volumes of ice cold ethanol and pelleted in a microfuge. The dried pellet was dissolved in 10 μl sterile diethyl pyrocarbonate [DEPC] treated water. The concentration of 1 μl in 500 μl of water was determined at 260 nm spectrophotometrically.

Preparation of samples for dot blots. Aliquots of each mRNA were taken to give 1800 ng of mRNA. The aliquots were incubated at 65°C for 15 min in the presence of 3 volumes of 6.15 M formaldehyde in 10X SSC. Samples were cooled to room temperature and diluted with 4.15M formaldehyde in 7.5X SSC to give a final concentration of 100 ng per 200 μl solution. Nitrocellulose filter was soaked in 10X SSC, airdried and placed in BRL Hybri Dot Manifold. A sample of 200 μl of each mRNA was applied under vacuum, the wells rinsed with 10X SSC and the filters airdried before being baked for 2 h at 80°C in a vacuum oven.

Preparation of samples for Northern blots. One set of P388-D1 mRNAs and one set of RAW mRNA's were run on gels; mRNA was transferred onto nitrocellulose by Northern blot[22,23]. Two μg of each of the mRNA's obtained from RFR-exposed and sham exposed cells at each test interval of time and temperature were denatured in 50% formamide, 2.2 M formaldehyde, 40 mM 3-[N morpholine]propane-sulfonic acid [MOPS], 10 mM sodium

acetate and 1 mM ethylene diaminetetraacetic acid [EDTA], heated at 60°C for 5 min, made 2.5% in glycerol and 0.025% in bromphenol blue and then loaded onto a 1% agarose-formaldehyde submarine gel (2.2 M) buffered with 40 mM Mops, 10 mM sodium acetate and 1 mM EDTA. The gel was immersed in the MOPS buffer without formaldehyde and run at 60 volts for 6 h. The gel was removed, inverted on 2 sheets of Whatman No. 3MM filter paper, overlaid with nitrocellulose paper and paper towels and blotted for 2 h using 20X SSC solution (1X SSC contains 0.15 M NaCl 0.015 M Na citrate). The filter was removed, air dried and baked for 2 h at 80°C under vacuum. Gels were stained with ethidium bromide to check efficiency of transfer. A faint band could be seen at 8.9 kb where the murine sarcoma virus probe was located. Approximately 4.5 ng of each probe was run on each gel as an internal check on hybridization.

Prehybridization and Hybridization. Prehybridization of the filters took place overnight at 42° C in heat sealed plastic bags. The prehybridization solution contained 50% formamide, 5X Denhardt's solution, 2.5 μg/ml sonicated salmon sperm DNA and 5X SSPE (1X SSPE contained 1 mM EDTA, 10 mM NaP and 0.18 M NaCl). Hybridization solution had the same composition as that for prehybridization, but with the addition of 0.1% SDS. After hybridization for 20 h at 42° C, the filters were washed in 2X SSC + 0.1% sodium dodecyl sulfate [SDS] four times at room temperature for 15 min per wash, followed by 2 washes in 0.1X SSC + 0.1% SDS at 55° C for 30 min per wash. Filters were then blotted to remove excess moisture, wrapped in plastic wrap and exposed to Kodak XAR film with or without intensifying screens for 24-48 h. Films were read on a Hoefer Scientific Instruments GS300 Scanning Densitometer.

Probes. Probes used were *v-fos*[24] selected as a probe since *c-fos* production has been shown to be induced by growth factors and has been shown to precede the activation of *c-myc* after stimulation by these factors, *v-myc*[26,27], *v-Ha-ras*[28] and a 1.45 kb Pst I fragment containing the LTR region, U3 and a small portion of U5 from the Mouse Mammary Tumor Virus (MMTV), which was used as a probe to look at expression of LTR mRNA before and after microwave exposure[29]. There are several lines of evidence indicating that the LTR regions of the retrovirus genome play a crucial role in controlling the level of transcription of the genome[30,31].

A probe for the 3611-murine sarcoma virus (MSV), an 8.9 kb EcoRI fragment, was further digested by restriction endonucleases BstE II and Xho I to give a 1.2 kb fragment containing sequences coding for *v-raf*[32]. Both the 8.9 kb fragment and *v-raf* were used as probes.

A probe for the 70,000 MW Human Heat Shock protein (hsp 70) was made by digesting the plasmid pUR.HS70 with EcoRI.Bam HI. The resulting 800 bp fragment contained 125 bp of pBR322. The pUR.HS70 plasmid was a gift of Dr. Joseph Nevins, Rockefeller University.

All probes were labelled with [^{32}P] dCTP by the oligonucleotide primer procedure of Feinburg and Vogelstein[33] to give approximately 1×10^9 dpm/μg. Before use probes were treated with 1/10 volume 1N NaOH for 10 min at 37° C then neutralized by addition of 1/10 volume 1N acetic acid.

RESULTS

For each set of RFR-exposed cells there is a set of sham-exposed cells giving a direct control both for temperature and RFR effects. As the temperature rose, it became apparent that with some probes hybridization signals became weaker for both sham-exposed

and RFR-exposed samples. Temperature affected RFR-exposed and sham-exposed cells equally with marked signal loss occurring between 42° and 45°C. This temperature effect did not appear to be the case with L5178Y cells with the *v-myc* or hsp70 probes where the 45°C signal was as strong as that at 42°C. Dot blots of L5178Y cells showed hybridization to *v-fos* at all temperatures with no consistent differences between the RFR-exposed and sham-exposed controls.

CHO cells showed no *v-fos* hybridizing material. Pilot studies on total RNA from non-exposed RAW and P388-D_1 cells indicated both cell types showed little *v-fos* hybridizing material.

Dot blots of L5178Y mRNA to the *v-myc* probe showed a strong hybridization signal, but no consistent differences between RFR-exposed and sham-exposed cells. mRNA from CHO cells showed a weak hybridization signal with no differences between RFR-exposed and sham-exposed cells.

mRNA from L5178Y and CHO cells showed a very weak hybridization to the *v-Ha-ras* probe and no consistent differences between expression of mRNA from the RFR-exposed or sham-exposed cells. RAW and P388-D1 cellular mRNA showed no hybridization to this probe.

The MMTV-LTR probe also showed very weak signals with mRNA from RFR-exposed or sham-exposed L5178Y or CHO cells. RAW total RNA showed no hybridization to this probe. Pilot studies with mRNA from unexposed P388-D1 cells suggested that strongly hybridizing material was present, but no results of RFR exposures are available.

mRNA from L5178Y cells showed a strong hybridization signal with both MSV and *v-raf* probes, although no significant differences were seen between the hybridization signals of mRNA derived from RFR-exposed cells and from sham-exposed cells (Table I). mRNA from CHO cells gave a weak signal with both RFR-exposed and sham-exposed samples with these MSV and *v-raf* probes. Pilot studies indicated unexposed cell mRNA from both RAW and P388 D1 cells strongly hybridized to these probes.

The heat shock protein probe, hsp70, hybridized to both L5178Y and CHO mRNA, but in neither case were there significant differences between signals of mRNA derived from RFR-exposed and sham-exposed cells. P388 D1 and RAW mRNAs were not tested with this probe.

DISCUSSION

The results of this study indicate that exposures of cultured rodent cells to 2450 MHZ

TABLE I. Ratios (RFR-exposed/sham-exposed) of signals detected by densitometer at designated times and temperatures using L5178Y cells and the MSV probes.

Time	*Temperature of RFR Exposure* 37°	40°	42°	45°
0	1.47 (4)[a,c]	0.75 (6)	1.56 (6)	0.59 (2)
3	0.67 (6)	1.22 (4)	1.14 (5)	2.55 (3)
5	1.32 (6)	1.59 (6)	0.86 (6)	1.39 (1)
10	1.21 (6)	0.55 (6)	0.66 (5)	1.02 (1)
15	1.19 (6)	1.69 (6)	1.68 (4)	
20	1.81 (4)	1.35 (4)		
30[b]	0.62 (2)	1.98 (2)	0.25 (2)	

[a]MSV and v-raf probe figures were combined to give greater numbers at each reading.
[b]In two experiments a 30 min reading was taken instead of a 20 min reading.
[c]Number in brackets denotes number of experiments per interval.

RF radiation at SAR of 103.5 ± 4.2 W/kg, and CHO cells at SAR 51.75 W/kg do not significantly change the levels of mRNA expression as determined by stringent hybridization conditions using DNA probes for *c-fos, c-myc, c-Ha-ras,* the LTR of mouse mammary tumor virus, p21 Sarc protein and *c-raf* and the 70 kD heat shock protein. Kirschmeier et al[34] showed that normal C3H10T½ mouse cells could be transformed by UV light, X-ray irradiation and chemical carcinogens and that they expressed increased levels of mRNA homologous to a probe specific for LTRs. Normal cells showed little or no hybridization to this probe. Our present study using RFR exposure did not show such increases in mRNA. To our knowledge this study is the first attempt to detect and quantify any relationships at the mRNA hybridization level between gene expression and exposure to RFR radiation. It appears that no direct relationship exists between the time-temperature condition used in these studies of a standardized RFR radiation exposure field and induction of effects on mRNA expression.

This study was conducted under very controlled temperature conditions. Thermal changes due to microwave exposure can cause changes in biological systems that can be interpreted as being due to the microwaves themselves. Kiel et al[35] reported this in their experiments with leukocytes. Oxidative metabolic activity of human peripheral mononuclear cells appeared to be significantly increased after exposure of the cells to microwaves when compared with the activity of incubator controls. When sham-treated controls were compared with the incubator controls a similar increase was seen, but when the sham-treated controls were compared with the microwave treated cells no significant differences were seen. It is therefore very important to treat sham-control and microwave-exposed cells in the same way, constantly adjusting the temperature to negate any increase due to the thermal effects of the microwaves. Thermal changes can affect results and can lead to incorrect conclusions. In this study sham-control and microwave-exposed samples were treated identically and each sample had its own built in control at each temperature and length of exposure. As the temperature increased we saw an increase in m-RNA levels of the heat stress protein probe in both sham-exposed and microwave-exposed samples as would be expected.

Further studies using different cell lines and longer exposure times may reveal sensitivities to RFR or it may be also that an outside stimulus, i.e., a hormone or chemical, is needed to alter the "normal" cell, thus making it more susceptible to exposure to microwave radiation[36].

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